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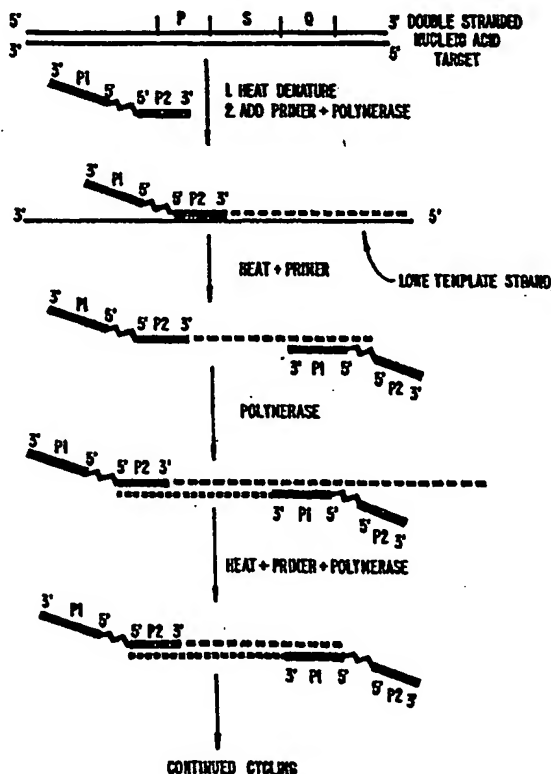
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(54) Title: AN EXPONENTIAL NUCLEIC ACID AMPLIFICATION METHOD USING A SINGLE PRIMER OF OPPOSING POLARITY

## (57) Abstract

This invention relates to the amplification of nucleic acid using single nucleic acid primer having two 3' ends.



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## AN EXPONENTIAL NUCLEIC ACID AMPLIFICATION METHOD USING A SINGLE PRIMER OF OPPOSING POLARITY

### CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

### FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable.

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### FIELD OF THE INVENTION

This invention relates to the amplification of nucleic acid using single nucleic acid primer having two 3' ends.

### BACKGROUND OF THE INVENTION

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The present invention describes a novel nucleic acid amplification method utilizing a unique primer structure referred to as a single opposing polarity primer. Many nucleic acid amplification technologies are now available. This method is distinct in that only a single primer addition is required for amplification. This new primer structure can be synthesized on a standard nucleic acid synthesizer using commercially available reagents. Besides all the advantages of other amplification technologies, use of the single opposing polarity primer has the advantages that only a single primer need be added to the amplification mix, that since the single primer has two sequence specificities these are added to the reaction in perfect balance, that the unique dual 3' end of the primer avoids 5' exonucleolytic degradation, and that the amplification products all have perfectly single stranded tails that may aid in product detection by hybridization.

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## SUMMARY OF THE INVENTION

This invention provides for a method for amplification of a target nucleic acid sequence using 3' enzymatic extension and a single amplification primer. The method comprises the steps of: (a) contacting in an aqueous solution the target nucleic acid having three defined regions said regions being a 5' P region, an intervening S region and a 3' Q region with an oligonucleotide primer comprising two 3' ends connected through a bond or linking composition wherein the first 3' end, 3P-1 is able to hybridize to the 3' Q region of the target nucleic acid sequence and the second 3' end 3P-2 is able to hybridize to the complement of the 5'P end of the target oligonucleotide sequence; and, (b) further contacting the solution of step i with a DNA polymerase that carryouts 3' DNA extension wherein said contacting is (a) under conditions in which the primer is able to hybridize to either the complement of the 3'Q region or the 5'P region of the target and (b) under conditions and with reagents where the polymerase is able to produce a copy of the target's S region. For cycling of the amplification method, the copy is separated from the template target sequence, the primer hybridized to both the original target and the 3' extension copy and additional copies of the S region synthesized by 3' extension of the primers using DNA polymerase. Separation may be achieved by heat denaturation or by a helicase enzyme.

A preferred polymerase is thermostable as it resists denaturation under temperatures required to denature duplexed nucleic acid.

The target nucleic acid may be a double stranded DNA.

The amplification method can use a primer wherein the 3'P-1 and 3'P-2 are linked by nucleotides. The method can also include an amplification primer is covalently linked to a detectable reporter.

The method of this invention can involve target nucleic acid that is diagnostic of an animal or plant pathogen and where the target nucleic acid is an animal or plant gene.

In addition to the method described above, this invention provides for nucleic acid amplification primers comprising nucleic acid sequences having two 3' ends where both ends complement different nucleic acid sequences of a target double stranded nucleic acid sequence where the target has three defined regions: a 5' P region, an intervening S region and a 3' Q region;

wherein the primer has a first 3' end, 3P-1 which is able to hybridize to the 3'Q sequence of the target nucleic acid sequence and a second 3' end, 3P-2 which is able to hybridize to the complement of a 5'P end of the target oligonucleotide sequence. The primers can be directed to any type of target and be labelled as described above in the method provided herein.

In addition to the method and primers, there is also provided aqueous solutions comprising the nucleic acid amplification primers described herein. The solution can also comprise the amplification reaction components described above such as thermostable DNA polymerases, helicase enzymes and assorted target nucleic acids including genes of any sort and pathogens that need to be diagnosed in medical or agricultural applications.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of the amplification system.

Fig. 2 is a single primer of opposing polarity exponential amplification of the *E. coli* GroEL gene.

Fig. 3 is a single primer of opposing polarity exponential amplification of the HIV RT gene.

#### DEFINITIONS

The term "amplification" refers to the duplication of a target nucleic acid while in its broadest context the term includes a single duplication, optimum use of the invention involves a series of repetitive steps that result in exponential duplication of a target nucleic acid.

The term "complement" refers to a strand of nucleic acid which is hybridized to, or which has the ability to hybridize to a reference strand of nucleic acid. An exact complement indicates that the every base in the reference strand matches the bases in the hybridizing strand with which it can form hydrogen bonds. An inexact complement means that at least one mismatched base is present between the reference strand and the hybridizing strand.

The term "covalently linked" refers to a bond between two distinct molecular species that is the result of shared electrons between constituent atoms of each species.

The term "single amplification primer" refers to a single molecular species which can bind to a target nucleic acid template sequence and directs the synthesis of a new nucleic acid strand complementary to the template strand.

5 The term "target" refers to any nucleic acid species which may hybridize with the single opposing polarity primer and which can be copied by a polymerase activity.

The term "3' enzymatic extension" refers to an activity carried out by a polymerase molecule in the addition of nucleotides to the 3' end of a nucleic acid molecule.

10 The term "thermostable DNA polymerase" refers to an enzymatic activity that can be isolated from a number of bacterial or mammalian sources which exhibits the ability maintain enzymatic activity in response to repeated cycles of heating and cooling.

## DESCRIPTION OF THE PREFERRED EMBODIMENT

### A. General Introduction:

15 The present invention relates to a new method for the amplification of nucleic acid sequences utilizing a single primer of unique structure. This amplification method is expected to have broad applicability in the areas of genetic research, diagnostics, and forensics. Methods for the production of the single amplification primer, reaction conditions, and diagnostic kits are described.

20 Many techniques for the amplification of nucleic acid sequences have been described. The best known is the Polymerase Chain Reaction (or PCR). Others include the Ligase Chain Reaction, transcription based nucleic acid amplification such as NASBA, and single primer amplification systems such as SPA. PCR and LCR both require temperature cycling to cause nucleic acid amplification, while NASBA is an isothermal reaction.

25 All nucleic acids have strand polarity. That is, sequences are directional defined by the chemical bond formed between adjacent bases within the nucleic acid strand. Thus, the 5' carbon of the sugar moiety of each nucleotide is linked to the 3' carbon of the sugar moiety in the adjacent nucleoside. Therefore, every natural nucleic acid strand has both a 5' and a 3' end. By convention, when drawing a nucleic acid structure, the upper strand usually is drawn in the 5' to 3' direction. The lower complementary strand would have the opposite polarity, or 3' to 5'. All polymerases

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require a free 3' hydroxyl group onto which nucleotides are added. Normally, when oligonucleotides are prepared for amplification reactions, they are synthesized having a single 5' to 3' polarity. Therefore, for an amplification reaction such as PCR, two oligonucleotides must be synthesized, each complementary to the opposite strands.

5 A novel aspect of this invention is that this amplification method requires only a single primer having two 3' ends. The single primer contains two sequence specificities defining the region of the target which is to be amplified. The two sequence specificities are arranged in opposite polarities and joined through their 5' ends as shown below:

10 3' - Sequence 1 — 5' — 5' — Sequence 2 - 3'

Since this structure has two 3' ends, it does not have a classical oligonucleotide structure which would normally have a single polarity sequence running 5' to 3'. The bond between both sequence specificities can be directly through a phosphodiester linkage, or may be through other spacer arms of varying length. The spacer arm itself may have functional groups useful in the analysis of the amplification products. In addition, each sequence specificity may contain detectable groups such as biotin. Sequences may also be introduced that would allow cloning of amplification products, or production of RNA transcripts corresponding to the amplified product.

15 The use of the single primer in an amplification reaction is shown in Figure 1. Because the single primer contains both sequence specificities, only one addition of this primer is required for amplification. In the first step, the template strand is denatured so that the primer may hybridize to its complement. The 3' end of the primer is then extended by a polymerase activity forming the complementary strand. In the next step, the two strands are once again denatured allowing binding of the second sequence specificity of the single primer. The 3' end can then be extended once again by the polymerase activity to complete the second strand. The product of one round of amplification will then have single strand tails. While the product of a standard amplification reaction will have a normal 5' to 3' polarity, the product of the amplification reaction using the single primer will have four 3' ends. Furthermore, in most amplification methods, each mole of product synthesized would require one mole of each primer, in the single primer reaction will require two moles of primer for each

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mole of double strand product. The single strand tails arise in the amplification method since all DNA polymerase activities work in a 5' to 3' direction only, therefore, no polymerase can read across the polarity change in the primer. As a result, in the course of the amplification, every product will have a single strand tail in the opposite polarity to the rest of the amplification product.

It may be expected that this amplification system might not yield discrete products, but rather a smear of concatameric products. This is because the product of the amplification has functional priming sequences on it's ends which can bind to both template strands yielding amplification products joined through the opposing primer. Multiple cycles would then lead to concatameric products of increasing length. This potential problem may be exacerbated in the later amplification cycles when the product template concentration is much higher, and the primer concentration is lower which would encourage product primer binding to both template strands. The data presented below demonstrates that this does not happen, or if it does happen, it constitutes a very minor secondary reaction. In fact, very discrete amplification products were obtained. Also, the presence of the second sequence specificity with opposite polarity does not interfere with the hybridization of the first sequence specificity.

In the methods described in this application, the use of the single opposing polarity primer is demonstrated using temperature cycling to effect strand denaturation. In other applications, any method to cause strand separation can be utilized including chemical methods and enzymatic activities such as that derived from helicase proteins.

This invention has the same features and utility as other amplification systems. Two important uses of amplification products are for cloning and for the generation of *in vitro* RNA transcripts.

Amplification products of the described process can be cloned if the central polarity break segment is removed. For cloning purposes, the opposing polarity primer is designed such that an appropriate restriction enzyme recognition sequence is inserted into the primer sequence on both sides of the polarity break. After the amplification reaction, the free 3' tail can be removed by restriction enzyme digestion.

RNA polymerase recognition sequences can likewise be incorporated into the primer for the generation of RNA transcripts from the amplification product. The RNA polymerase binding site is generally 20 - 30 bases long. In the opposing polarity primer, this sequence would be positioned on one side of the polarity break

corresponding to the strand for RNA is to be made. RNA transcripts prepared this way are convenient research tools, or can be used to prepare controls for diagnostic kits.

Some additional features of the amplification system described in this application include the following. For diagnostic assays, only a single primer addition is required, which has built in both sequence specificities in a perfect balance. Also, some thermostable polymerases do have 5' - 3' nuclease activity which can result in the removal of 5' terminal groups, an activity which would be avoided in this system. In addition, products of the single primer amplification have single stranded tails on completed products which may be useful in hybridization detection of the reaction products.

#### B. Production of the Amplification Primer

The single opposing polarity primer may easily be prepared on a standard DNA synthesizer. In standard DNA synthesis, 3' nucleotide phosphoramidites are added to the 3' most base of the sequence being synthesized in a step-wise fashion. To change the backbone polarity, 5' phosphoramidites are also available which can be added on the synthesizer in the opposite direction. Therefore, one would synthesize the first sequence specificity using the standard 3' nucleotide phosphoramidites. When the 5' end of that sequence is reached, the 5' phosphoramidites are then added to complete the second sequence specificity.

A second method would be chemical linkage of two oligonucleotides with 5' modifications. These modifications include but are not limited to 5' amine, 5' sulfhydryl, 5' phosphate, and 5' maleimide groups. Modifying reagents could be bifunctional or homofunctional. To use bifunctional reagent by way of example, the first oligonucleotide could be synthesized directly having a 5' sulfhydryl group. The second oligonucleotide could be synthesized having a 5' amine group. The 5' amine could be modified to a sulfhydryl specific 5' iodo- group using the bifunctional cross-linking agent Succinimidyl(4-iodoacetyl) Amino Benzoate (SIAB). The sulfhydryl and iodo- modified oligonucleotides could be mixed and allowed to react. It would then be necessary to purify the opposing polarity primer from the reaction mix by HPLC, size exclusion chromatography, or gel electrophoresis. Homofunctional cross-linking reagents could likewise be used. For example, the first oligonucleotide could be reacted with a large excess of the reagent cyanuric chloride. Since the reaction products will include

collected and resuspended in water or buffer and stored at -70 C. In an alternative method, RNA is selectively precipitated from the guanidine thiocyanate lysate with 0.5 vol. ethanol. The precipitate is collected by centrifugation and resuspended in water for storage at -70 C.

5                   iv. Plasmid DNA

Bacterial cells carrying plasmid DNA are collected by centrifugation. The cells are resuspended in buffer containing lysozyme. After digestion, the cells are lysed with an alkaline SDS solution which also denatures the bacterial genomic DNA and plasmid DNA. The suspension is then neutralized causing renaturation of the plasmid  
10 DNA and precipitation of the genomic DNA. The precipitate is removed by centrifugation, and the plasmid DNA in the supernatant is precipitated with ethanol. The plasmid DNA is suspended in water or buffer and then stored at -70 C.

**D. Amplification Reaction Components and Conditions**

i. Primer Design

15                   The dual opposing polarity primer is designed much like primers for other amplification systems. The length of each recognition sequence in the primer is chosen to be, but not limited to, a range of sizes from 15 to 30 bases. The preferred GC content is 50% but other compositions may be used as well. Modified bases such as those containing biotin or chemically reactive groups such as free primary amines may be  
20 incorporated into the primer. Sterile distilled water is added to the primer to make a final concentration of 100uM. The concentrated primer solution is stored at -20 C.

ii. Reaction Conditions

A typical reaction will contain the single opposing polarity primer at a final concentration of 3uM, buffer salts, 20mM Tris-HCl (pH8.4), 50mM KCl, 2mM  
25 MgCl<sub>2</sub>, and 200uM dNTPs, the template, and the DNA polymerase. Amplification reactions are run using a standard DNA thermocycler.

The magnesium concentration is critical for efficient amplification. The concentration must be above 1.5mM. For this amplification reaction, 2mM is chosen as a sufficient concentration, but for certain templates and primers, it may be necessary to  
30 titrate the magnesium concentration to the optimum concentration.

Other reaction conditions may affect the amplification efficiency. The amount of salt added to the reaction (KCl) maintains a sufficient ionic strength for

binding of the primer during amplification. However, salt concentrations above 100mM may adversely affect the polymerase activity. The concentration of the nucleotides must be relatively low (0.2mM). If the concentration is too high, misincorporation of nucleotides may result in sequence errors. The primer concentration must be controlled since if the concentration is too high, then mis-priming or non-specific priming may result.

For the single opposing polarity primer amplification reaction, it is necessary to use a thermostable polymerase. A variety of thermostable polymerases are now available commercially including but not limited to Taq, Pfu, and Pwo. The amount of added polymerase can influence the specificity of the amplification reaction. Too much polymerase can lead to non-specific amplification, while too little will decrease the yield of product. For example, for a 100ul reaction mix, 1 unit of Taq DNA polymerase is sufficient for specific amplification with good yield of product.

When using the single opposing polarity primer to amplify a sequence from an RNA template, it is first necessary to copy the RNA strand into DNA utilizing a reverse transcriptase activity. In this case, a 20ul reaction mix contains 20mM Tris-HCl (pH8.4), 50mM KCl, 2mM MgCl<sub>2</sub>, and 1mM dNTPs, at least 1uM single opposing polarity primer, template RNA, and sufficient reverse transcriptase activity. Several reverse transcriptases are commercially available. Commonly used is AMV reverse transcriptase or MMLV reverse transcriptase. The reverse transcription reaction is incubated for 1 hour at 42 C. Then, the reaction mix is diluted with water and buffer to maintain the salt concentration and primer concentration, while diluting the dNTPs to 0.2mM. The reaction is then processed by thermal cycling.

There are three segments to a typical thermal profile, the melting temperature in which the template is converted to single strands, the annealing temperature in which the primer will bind to the template, and the extension temperature, in which the polymerase has maximum activity for primer extension. The best thermal profile is determined empirically and is based on the sequence of each segment of the single opposing polarity primer. For example, from the sequence, one can determine the T<sub>m</sub> of each segment of the primer. Then, the annealing temperature can be chosen as about 5 degrees below the T<sub>m</sub>. The number of cycles is chosen based on the amount of input template, and the level of sensitivity required.

### iii. Helicase Mediated Amplification

An ideal use of the single opposing polarity primer is the design of an isothermal amplification system utilizing helicase activity to separate DNA strands instead of using heat. Helicase proteins separate double stranded DNA at the expense of hydrolysis of ATP. Helicase binds to the 3' end of single stranded DNA with high affinity and translocate down the strand. A number of helicase proteins have been identified and studied. It has been found that helicases oligomerize when they bind to a nucleic acid. Some, such as the *E. coli* DnaB protein and the SV40 large T antigen, form hexamers when bound. A hexamer due to its size would require a large binding site making these proteins less suitable for an amplification mediating protein. Some activities such as the SV40 T antigen also possess an RNA helicase activity. Others such as the *E. coli* Rep protein, the *E. coli* UvrD protein, and the HeLa helicase form dimers which require much smaller binding sites. The mechanism of action of some of these proteins has been studied in detail. The Rep helicase isolated from *E. coli* has been shown to be able to "jump" polarities, a situation that would be encountered using the opposing polarity primer. Since every amplification product will have a free single stranded tail, helicase activity may bind to the tail and translocate across the polarity change to separate strands of newly synthesized amplification products. The utility of the helicase mediated amplification system will be further enhanced by the use of thermostable helicase protein which can be isolated from a number of thermophilic bacteria. Possible sources are *Thermus aquaticus* and certain *Thermophilus* species. This would allow the amplification to be done at higher temperature which would increase the specificity of primer binding.

### iv. Detection of Amplification Products

Amplification products can be detected by a number of methods. The simplest is by analyzing a portion of the amplification product by gel electrophoresis. From the known sequence, the size of the specific product can be predicted. Non-specific amplification products would appear as bands of weaker intensity at varying mobilities from the specific product. Gel electrophoresis is particularly convenient when amplification conditions are being developed, since it is relatively easy to determine the specificity of the amplification by the yield of specific product and lack of spurious products. Amplification products from the single opposing polarity primer amplification reaction have slightly slower mobilities than amplification products without the single

strand tails. However, particularly when running diagnostic assays, it is best to confirm the identity of the amplification product using hybridization analysis. A convenient method is based on forming a sandwich between the amplification product, a capture oligonucleotide linked to a solid phase, and a specific signal oligonucleotide. The signal oligonucleotide may contain a reporter group which will be recognized by a binding molecule conjugated to an enzymatic activity. For example, the signal oligonucleotide may have a biotin group which would be recognized by a streptavidin-horseradish peroxidase conjugate. Alternatively, a signal oligonucleotide may not be needed if the amplification product is labeled with biotin during the amplification, or if the primer itself is labeled with biotin. In either event, the presence of a successful amplification can be determined by incubating the solid phase in contact with a colorimetric substrate and measuring the amount of color generated.

To maintain specificity of the hybridization reaction, the hybridizations may be carried out at elevated temperatures, or in the presence of chaotropic salts. Salt concentration and temperature combine to define the stringency of the hybridization. In general, low salt concentration together with high temperature would be considered high stringency conditions. High stringency conditions would be used under conditions where an exact match between the capture probe and the amplification product is desired. For example, when differentiating between amplification products differing by a single base pair mismatch, high stringency is required. On the other hand, in some instances, one may wish to detect hybridization in which one or more mismatches are allowed. Low or relaxed stringency conditions consists of high salt and lower temperatures. These conditions would allow hybridization analysis of identical genes between closely related species. Alternatively, if the hybridization is done in the presence of a chaotropic salt such as guanidine thiocyanate, then stringency may be controlled by either high concentrations of chaotrope (high stringency), or low concentrations of chaotrope (low stringency).

In DNA hybridization analysis, one problem that is frequently encountered is renaturation of the amplification products before they may be hybridized to the capture oligonucleotide. The single opposing polarity primer amplification products may offer an advantage when only detection of a single species is desired. This is due to the single strand tails present on the amplification products. For example, the amplification product may be labeled with biotin during the amplification reaction. Then, either the

complement of the sequence of either tail may be used to capture the amplification product. Since the tails are always single stranded, the lack of strand competition should result in a very sensitive detection step.

#### E. Kits

5           The single opposing polarity primer amplification reaction is best used in the design of diagnostic kits. The primer could be supplied in such a kit in either of two forms, as a separate reagent to be added to a reaction mix for amplification of a specified target, or in an optimized reaction buffer ready to be added to a sample with only addition of the enzyme being otherwise required. As an example, a kit for the detection  
10 of *E. coli* would contain: 1. 2X reaction mix containing a buffer component, the single primer, magnesium salts, and deoxynucleotide triphosphates. This reagent may be supplied pre-dispensed in appropriate tubes ready to use 2. Sterile water to dilute the sample 3. Thermostable polymerase which may be part of the 2X reaction mix 4. Detection reagents consisting of capture oligonucleotides in a preadjusted buffer, and a  
15 reagent plate containing the remainder of the sandwich hybridization reagents.

#### EXAMPLES

The examples provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

##### 20   Example 1

PCR has been used to detect *E. coli* using two primers directed towards the GroEL gene. The primers have the following sequences

5' AAA CGT GGT ATC GAC AAA GC<sup>3'</sup>

5' CGG TCG AAC TGC ATA CCT TC<sup>3'</sup>

25   A single primer was synthesized having the sequence

3' CTT CCA TAC GTC AAG CTG GC<sup>5'</sup> -p- 5' AAA CGT GGT ATC GAC AAA GC<sup>3'</sup>

For comparison, a two primer PCR reaction was prepared using the primers listed above.

The reaction conditions were in 100ul total volume 20mM Tris-HCl (pH8.4), 50mM KCl, 2mM MgCl<sub>2</sub>, and 200uM dNTPs. For the PCR reaction, each of the two primers was added to a final concentration of 1uM. For the single primer amplification, the amount of added primer was varied. Each reaction was run using 1 unit of Taq polymerase (Life Technologies) and 1ug of DNA. Samples were overlaid with mineral oil and thermocycled using the following program: 1min. at 94°C, 1 min at 50°C, 1 min at 72°C for 35 cycles, a 5 min final extension at 72°C, and a 4°C soak.

The results of the experiment are presented in Fig. 2. A control tube is run without the addition of template to assess the presence of background amplification. The PCR products using two oligonucleotide primers yields a single band as expected when the template DNA is included in the reaction mix. Without template, no product is obtained. Likewise, the single primer reaction yields a single amplification product that migrates more slowly than the PCR product. This is due to the presence of the single strand tails on the single primer amplification products. The yield of product is dependent on the input amount of the single opposing polarity primer. By visually comparing the intensities of the bands obtained by both amplification methods, it is apparent that at least 2uM single opposing polarity primer is required to yield similar amounts of amplification product as a standard PCR reaction which has 1uM of each primer present. 3uM single opposing polarity primer seems to yield more amplification product.

These results indicate that the single opposing polarity primer functions with similar efficiency to an equivalent PCR reaction. These results further suggest that the presence of the polarity break within the single primer does not interfere with primer binding or 3' extension by the polymerase.

#### Example 2

Detection of HIV sequences was accomplished using primers directed towards the reverse transcriptase gene. Primers were prepared for a standard PCR amplification, as well as a single primer for the method used in this application. The sequences are:

WHAT IS CLAIMED IS:

- 1                   1.    A method for amplification of a target nucleic acid sequence using  
2   3' enzymatic extension and a single amplification primer comprising:  
3                   i.    contacting in an aqueous solution the target nucleic acid having  
4   three defined regions said regions being a 5' P region, an intervening S region and a 3'  
5   Q region with an oligonucleotide primer comprising two 3' ends connected through a  
6   bond or linking composition wherein the first 3' end, 3P-1 is able to hybridize to the 3'  
7   Q region of the target nucleic acid sequence and the second 3' end 3P-2 is able to  
8   hybridize to the complement of the 5'P end of the target oligonucleotide sequence; and,  
9                   ii.   further contacting the solution of step i with a DNA polymerase  
10   that carryouts 3' DNA extension wherein said contacting is (a) under conditions in which  
11   the primer is able to hybridize to either the complement of the 3'Q region or the 5'P  
12   region of the target and (b) under conditions and with reagents where the polymerase is  
13   able to produce a copy of the target's S region.
- 1                   2.    A method of claim 1 wherein the copy is separated from the  
2   template target sequence, the primer hybridized to both the original target and the 3'  
3   extension copy and additional copies of the S region synthesized by 3' extension of the  
4   primers using DNA polymerase.
- 1                   3.    A method of claim 2 wherein the separation of the template target  
2   sequence and the copy is by heat denaturation.
- 1                   4.    A method of claim 1 wherein the polymerase is thermostable.
- 1                   5.    A method of claim 2 wherein the template target sequence and the  
2   extension copy are separated by a helicase enzyme.
- 1                   6.    A method claim 1 wherein the target nucleic acid is a double  
2   stranded DNA.

- 1                   7.     A method of claim 1 wherein the 3'P-1 and 3'P-2 are linked by  
2     nucleotides.
- 1                   8.     A method of claim 1 wherein the amplification primer is covalently  
2     linked to a detectable reporter.
- 1                   9.     A method of claim 1 wherein the target nucleic acid is diagnostic of  
2     an animal or plant pathogen.
- 1                   10.    A method of claim 1 wherein the target nucleic acid is an animal or  
2     plant gene.
- 1                   11.    A nucleic acid amplification primer comprising nucleic acid  
2     sequences having two 3' ends where both ends complement different nucleic acid  
3     sequences of a target double stranded nucleic acid sequence where the target has three  
4     defined regions: a 5' P region, an intervening S region and a 3' Q region;  
5                    wherein the primer has a first 3' end, 3P-1 which is able to hybridize to  
6     the 3'Q sequence of the target nucleic acid sequence and a second 3' end, 3P-2 which is  
7     able to hybridize to the complement of a 5'P end of the target oligonucleotide sequence.
- 1                   12.    An aqueous solution comprising a nucleic acid amplification primer  
2     comprising nucleic acid sequences having two 3' ends where both ends complement  
3     different nucleic acid sequences of a target double stranded nucleic acid sequence where  
4     the target has three defined regions: a 5' P region, an intervening S region and a 3' Q  
5     region;  
6                    wherein the primer has a first 3' end, 3P-1 which is able to hybridize to  
7     the 3' Q sequence of the target nucleic acid sequence and a second 3' end, 3P-2 which is  
8     able to hybridize to the complement of a 5'P end of the target oligonucleotide sequence.
- 1                   13.    A solution of claim 13 which further comprises a thermostable  
2     DNA polymerase.
- 1                   14.    A solution of claim 13 which further comprises a helicase enzyme.

1                   15.    A solution of claim 13 wherein the target nucleic acid is diagnostic  
2   of an animal or plant pathogen.

1                   16.    A solution of claim 13 wherein the target nucleic acid is an animal  
2   or plant gene.

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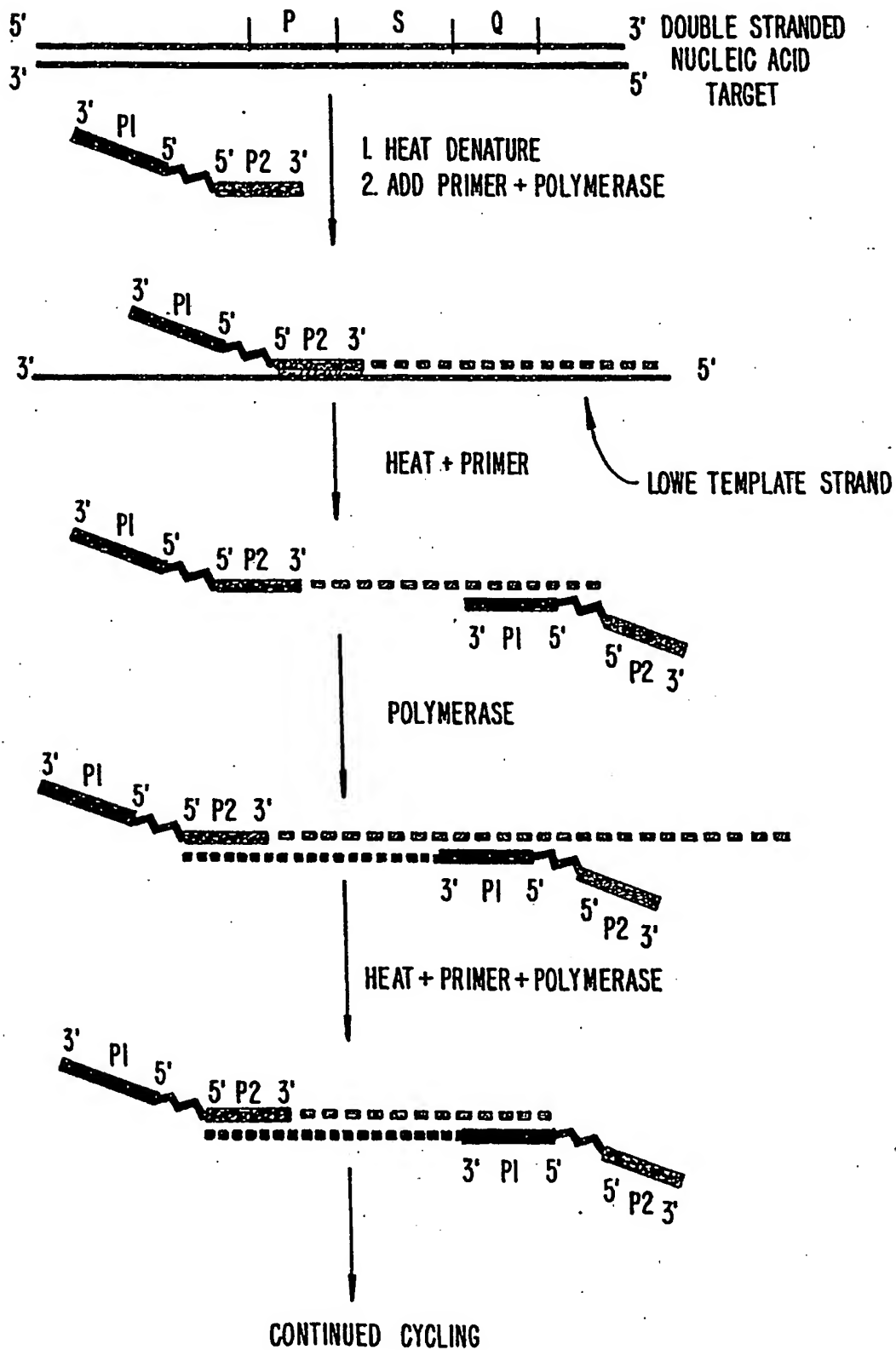


FIG. 1.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/00834

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C12P 19/34; C07H 21/00  
US CL : 435/6; 91.2; 536/24.33

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 91.2; 536/24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,683,202 A (MULLIS) 28 July 1987 (07/28/87), see entire document, especially column 4 lines 24-66.	1-16
Y	US 5,508,178 A (ROSE et al) 16 April 1996 (04/16/96), see entire document.	1-16
Y	US 5,043,272 A (HARTLEY) 27 August 1991 (08/27/91), see entire document.	5,13
Y	US 5,610,017 A (GUDIBANDE et al) 11 March 1997 (03/11/97), see entire document, especially column 2, lines 55-59.	8
Y	US 5,612,199 A (WESTERN et al.) 18 March 1997 (03/18/97), see entire document especially abstract.	1-16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* "A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"B" earlier document published on or after the international filing date	"X" documents of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"A" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

26 FEBRUARY 1999

Date of mailing of the international search report

25 MAR 1999

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/00834

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, WPIDS, BIOSIS, MEDLINE, CANCERLIT, BIOTECHDS, LIFESCI, CAPLUS, EMBASE

search terms: amplification, per, single amplification primer, dual, double, polarity, modify, crosslink, phosphoramidites, two  
3 prime, ends, nucleic acid regions, helicase, reporter

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